
Mechanisms of Signal Transduction:

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# Critical Role of Phospholipase Cγ1 in the Generation of $H_2O_2$-evoked $[Ca^{2+}]_i$ Oscillations in Cultured Rat Cortical Astrocytes*[S]

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**Reactive oxygen species, such as the superoxide anion, $H_2O_2$, and the hydroxyl radical, have been considered as cytotoxic by-products of cellular metabolism. However, recent studies have provided evidence that $H_2O_2$ serves as a signaling molecule modulating various physiological functions. Here we investigated the effect of $H_2O_2$ on the regulation of intracellular $Ca^{2+}$ signaling in rat cortical astrocytes. $H_2O_2$ triggered the generation of oscillations of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in a concentration-dependent manner over the range 10–100 μM. The $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations persisted in the absence of extracellular $Ca^{2+}$ and were prevented by depletion of intracellular $Ca^{2+}$ stores with thapsigargin. The $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations were not inhibited by pretreatment with ryanodine but were prevented by 2-aminoethoxydiphenyl borate and caffeine, known antagonists of inositol 1,4,5-trisphosphate receptors. $H_2O_2$ activated phospholipase C (PLC) γ1 in a dose-dependent manner, and U73122, an inhibitor of PLC, completely abolished the $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations. In addition, RNA interference against PLCγ1 and the expression of the inositol 1,4,5-trisphosphate-sequestering "sponge" prevented the generation of $[Ca^{2+}]_i$ oscillations. $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations and PLCγ1 phosphorylation were inhibited by pretreatment with dithiothreitol, a sulfhydryl-reducing agent. Finally, epidermal growth factor induced $H_2O_2$ production, PLCγ1 activation, and $[Ca^{2+}]_i$ increases, which were attenuated by *N*-acetylcysteine and diphenyleneiodonium and by the overexpression of peroxiredoxin type II. Therefore, we conclude that low concentrations of exogenously applied $H_2O_2$ generate $[Ca^{2+}]_i$ oscillations by activating PLCγ1 through sulfhydryl oxidation-dependent mechanisms. Furthermore, we show that this mechanism underlies the modulatory effect of endogenously produced $H_2O_2$ on epidermal growth factor-induced $Ca^{2+}$ signaling in rat cortical astrocytes.**

$H_2O_2$ is a member of the reactive oxygen species (ROS),[6] which cause oxidative damage to cellular components such as lipids, nucleic acids, and proteins. Therefore, $H_2O_2$ has generally been considered to be cytotoxic and hazardous to living organisms. However, a growing body of evidence suggests that $H_2O_2$ serves as an intracellular signaling molecule modulating various physiological functions (1). Cells possess mechanisms that can rapidly synthesize and destroy $H_2O_2$ in response to receptor stimulation. For example, stimulation of membrane receptors of various growth factors, such as transforming growth factor-β1, platelet-derived growth factor, and epidermal growth factor (EGF) triggers the rapid and transient production of $H_2O_2$ (2–5). $H_2O_2$ generated in response to receptor stimulation has been shown to play an important role in regulating various normal cell functions, such as cell proliferation, platelet aggregation, and vasodilation (6–8). In addition to this, exogenous addition of $H_2O_2$ at low concentrations affects the functions of various ion channels and other proteins involved in signal transduction (8–10). Therefore, $H_2O_2$ fulfills the prerequisites for being considered as a genuine intracellular messenger.

Recently, a great deal of attention has focused on the sensitivity of the mechanisms responsible for $Ca^{2+}$ mobilization in response to changes in the cellular redox state. $Ca^{2+}$ plays a pivotal role in the regulation of a diverse range of cellular functions, such as muscle contraction, secretion, synaptic plasticity, cell proliferation, and cell death (11). Many hormones and neurotransmitters increase intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) by mobilizing $Ca^{2+}$ from intracellular stores and by inducing an influx from the extracellular space (12, 13). $H_2O_2$ has been shown to enhance the activity of L-type $Ca^{2+}$ channels (10). Peroxide can also stimulate the mobilization of $Ca^{2+}$ in many cell types by modifying $Ca^{2+}$ release channels, such as TRPM2 (14), ryanodine receptors (15), and inositol 1,4,5-trisphosphate ($IP_3$)-dependent $Ca^{2+}$ channels (16). In addition, $H_2O_2$ can modify the activity of $Ca^{2+}$ pumps involved in $Ca^{2+}$ homeostasis, such as the sarcoendoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) (17) and plasma membrane $Ca^{2+}$-ATPase (17). Furthermore, enzymes involved in $Ca^{2+}$ signaling pathways, such as phospholipase C (PLC) γ1 (18) and phospholipase D (19) are also targets. However, most of the previous studies employed high concentrations of $H_2O_2$, and it is questionable whether such diverse actions of $H_2O_2$ on

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[6] The abbreviations used are: ROS, reactive oxygen species; PLCγ1, phospholipase Cγ1; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; $IP_3$, inositol 1,4,5-trisphosphate; EGF, epidermal growth factor; SERCA, sarcoendoplasmic reticulum $Ca^{2+}$ ATPase; PMCA, plasma membrane $Ca^{2+}$ ATPase; PLCβ1, phospholipase C β1; DTT, dithiothreitol; DPI, diphenyleneiodonium; NAC, *N*-acetylcysteine; DCF, 2′,7′-dichlorofluorescein diacetate; Prx II, peroxiredoxin type II; GFP, green fluorescent protein; siRNA, small interfering RNA; PSS, physiological salt solution; 2-APB, 2-aminoethoxydiphenyl borate; MEM, minimum essential medium; RNAi, RNA interference.

calcium signaling also occur under normal physiological conditions. Therefore, it will be of great value to identify the target molecules modulated by physiologically relevant concentrations of $H_2O_2$.

Astrocytes, the major glial cell type in the mammalian brain, participate in a variety of important functions in the central nervous system. As in virtually all other cell types, astrocytes also use $Ca^{2+}$ signaling to mediate a large spectrum of physiological responses. Elevation of $[Ca^{2+}]_i$ in response to stimulation of various receptors causes the release of neurotransmitters, such as glutamate and ATP, and plays an important role in the exchange of information with neurons and the regulation of local blood flow (20, 21). In contrast to $Ca^{2+}$ signaling, much less attention has been given to redox signaling in astrocytes. Because ROS are involved in the pathogenesis of neurodegenerative diseases and astrocytes have been shown to possess high antioxidant activities, many studies have focused on the protective roles of astrocytes against oxidative stress-induced neuronal cell death (22–24). However, despite the lack of information about the physiological roles of ROS in astrocytes, NADPH oxidase was shown to be involved in the generation of $H_2O_2$ and cell survival in this cell type (25). Given the widespread involvement of $H_2O_2$ in modulating $Ca^{2+}$ signaling cascades, it is tempting to speculate that astrocytes may also use redox signaling to modify their $Ca^{2+}$ signaling.

Therefore, in the present study, we sought to investigate the roles of $H_2O_2$ in $Ca^{2+}$ signaling in cultured rat astrocytes. Our results indicate that a low, physiologically relevant concentration of $H_2O_2$ (30 μM) induces $[Ca^{2+}]_i$ oscillations in a PLCγ1- and $IP_3$-dependent manner. In addition, $H_2O_2$ produced endogenously by EGF receptor stimulation is involved in the modulation of $Ca^{2+}$ signaling in rat astrocytes.

## EXPERIMENTAL PROCEDURES

*Materials*—$H_2O_2$, dithiothreitol (DTT), thapsigargin, 2-aminoethoxydiphenyl borate (2-APB), ryanodine, *N*-acetylcysteine (NAC), diphenyleneiodonium (DPI), caffeine, histamine, EGF, U73122, U73343, and 2′,7′-dichlorofluorescein diacetate (DCF) were purchased from Sigma. Minimum essential medium (MEM) containing 100 mg/liter sodium succinate and 75 mg/liter succinic acid, trypsin-EDTA, Opti-MEM, penicillin, streptomycin, and fetal bovine serum were purchased from Invitrogen. Fura-2-acetoxymethyl ester was purchased from Teflabs (Austin, TX). All other chemicals were of reagent grade. The polyclonal antibody against PLCγ1 and monoclonal antibody against phosphotyrosine (PY783) of PLCγ1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-PLCβ1 polyclonal antibody was kindly provided by Dr. Shmuel Muallem (University of Texas Southwestern Medical Center, Dallas).

*Cell Cultures*—Primary cultures of cortical astrocytes were prepared from neonatal (12–24 h) Wistar rats. Briefly, cortices were dissected, and the tissues were minced and mechanically dissociated. Then the isolated cells were plated on 60-mm culture dishes and maintained at 37 °C in a humidified 5% $CO_2$ and 95% air for 2–3 weeks. For $[Ca^{2+}]_i$ measurements, cells were cultured on 0.01% poly-L-lysine-coated cover glasses in 60-mm dishes for 7–10 days. The culture medium consisted of MEM supplemented with 2 mM glutamine, 25 mM glucose, 100 μg/ml penicillin, 25 ng/ml streptomycin, and 10% fetal bovine serum. The culture medium was replaced every 3 days. Cells were serum-starved for 2 days before each experiment.

*Expression of $IP_3$ Sponge and Peroxiredoxin Type II (Prx II)*—Astrocytes were transiently transfected with a green fluorescent protein (GFP)-tagged high affinity (R441Q) or low affinity (K508A) $IP_3$-sequestering sponge (26), or were cotransfected with Prx II (1 μg/ml; kindly provided by Professor S. W. Kang, Ewha Womans University, Seoul, Korea) and eGFP-N1 (1.2 μg/ml; Clontech) using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. Cells were incubated for 48 h at 37 °C, in a 5% $CO_2$ atmosphere with saturated humidity to allow expression of the construct before the experiment. The expression of each protein was confirmed by GFP fluorescence.

*Transfections of siRNA-PLCγ1*—Construction of a small interfering RNA (siRNA) for PLCγ1 (siRNA-PLCγ1) was described before (27). The pSUPER vector for siRNA was purchased from OligoEngine (Seattle, WA). Cells were cotransfected with siRNA-PLCγ1 (1 μg/ml) and eGFP-N1 (1.2 μg/ml) using Lipofectamine 2000 reagent and cultured for 48 h in serum-free MEM. Depletion of endogenous PLCγ1 by siRNA was confirmed by immunoblot or GFP fluorescence.

*Western Blot Analysis*—Astrocytes transfected with or without siRNA-PLCγ1 and eGFP-N1 were stimulated with $H_2O_2$ or EGF for the indicated times in the physiological salt solutions (PSSs) containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose (pH 7.4). Cells were then lysed at 4 °C in lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris, 10 mM NaF, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 mM leupeptin, and 1 mM sodium orthovanadate) and centrifuged at 12,000 rpm for 10 min at 4 °C. The samples were subjected to SDS-PAGE and subsequently transferred to nitrocellulose membranes. The membranes were incubated with specific antibodies against PLCγ1, phosphospecific tyrosine 783, PLCβ1, and actin, and the proteins were detected by ECL (Amersham Biosciences). The intensity of bands was quantified using MetaMorph Analysis System (Universal Imaging Co., Downingtown, PA).

*$[Ca^{2+}]_i$ Measurements*—For $[Ca^{2+}]_i$ measurements, attached cells were loaded with fura-2 by incubation with 3.5 μM fura-2-acetoxymethyl ester in PSS equilibrated with 100% $O_2$ for 40 min at room temperature. The cells were then washed twice and rested for at least 20 min before use. The fura-2-loaded cells were mounted on the stage of an inverted microscope (Nikon, Tokyo, Japan) for imaging. The cells were superfused at a constant perfusion rate with the PSS. In $Ca^{2+}$-free solutions, $CaCl_2$ was omitted, and 1 mM EGTA was added. The excitation wavelength was alternated between 340 and 380 nm, and the emission fluorescence was monitored at 510 nm with a CCD camera using MetaFluor system (Universal Imaging Co., Downingtown, PA). Fluorescence images were obtained at 4-s intervals. Background fluorescence was subtracted from the raw signals at each excitation wavelength, and the values of $[Ca^{2+}]_i$ were calculated using the equation described previously (28).

*ROS Imaging*—ROS levels were measured using the fluorescence probe DCF. In brief, cells were incubated for 5 min in the presence of 5μg/ml DCF and washed in Hanks' balanced salt solution. DCF fluorescence was measured using a confocal laser-scanning microscope (Leica, Buffalo, NY) with an excitation wavelength at 488 nm and an emission at 525 nm. To avoid photo-oxidation of DCF, the fluorescence images were collected using a single rapid scan, and identical settings were used for all samples.

*Data Analysis*—The results are presented as mean ± S.E. Statistical analysis was performed by unpaired Student's *t* test. *p* values lower than 0.05 were considered to be statistically significant.

## RESULTS

*$H_2O_2$ Mobilizes $Ca^{2+}$ in Cultured Rat Cortical Astrocytes in a Concentration-dependent Manner*—The effect of $H_2O_2$ on $Ca^{2+}$ mobilization was examined in fura-2-loaded cultured rat astrocytes. Exposure of the cells to $H_2O_2$ at concentrations lower than 3 μM failed to increase $[Ca^{2+}]_i$ at least for 20 min (Fig. 1*A*). However, 10 μM $H_2O_2$ was shown to induce $[Ca^{2+}]_i$ oscillations in 34.7 ± 8.4% of the 18 tested cells, and the

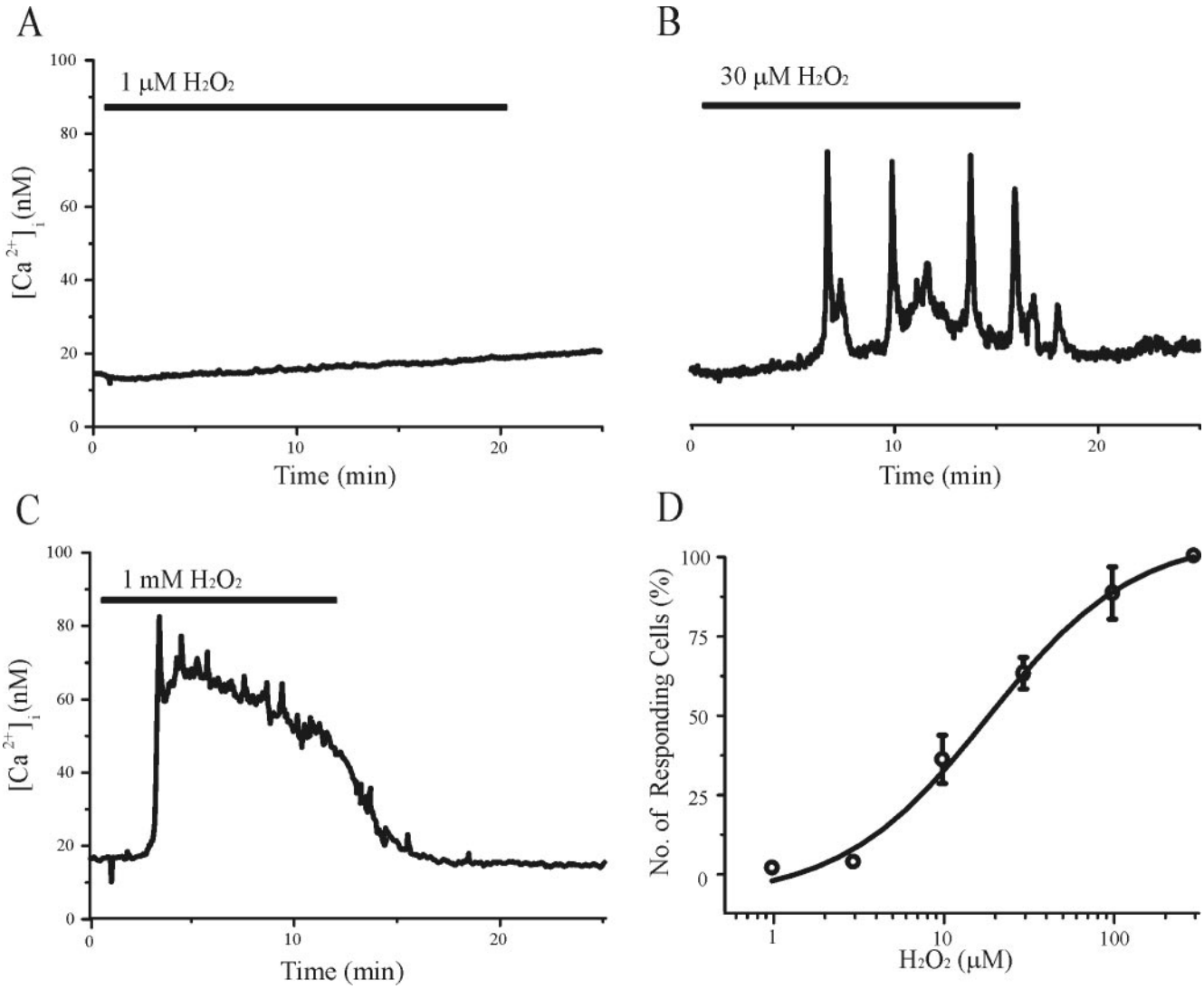


FIGURE 1. **$H_2O_2$ mobilizes $Ca^{2+}$ in a dose-dependent manner in cultured rat cortical astrocytes.** Cells were loaded with fura-2 as described under "Experimental Procedures," and changes in $[Ca^{2+}]_i$ were measured using ratiometric fluorescence imaging. Cells were exposed to 1 μM (*A*), 30 μM (*B*), or 1 mM (*C*) $H_2O_2$. For each panel, the trace is representative of 18–20 cells in four or five independent experiments. *D*, the number of cells responding to $H_2O_2$ is plotted as a function of the added $H_2O_2$ concentrations (1, 3, 10, 30, 100, and 300 μM). Data are expressed as the percentage of responding cells. Results are depicted as mean ± S.E.

number of the responding cells increased concentration-dependently (60.9 ± 5.8% for 30 μM, 84.9 ± 10.2% for 100 μM, and 93.1 ± 1.7% for higher than 300 μM $H_2O_2$; Fig. 1*D*, $n = 18–20$).

In general, as shown in Fig. 1, *B* and *C*, concentrations of $H_2O_2$ less than 30 μM induced oscillations of $[Ca^{2+}]_i$, whereas doses higher than 300 μM caused spike and plateau type $[Ca^{2+}]_i$ increases. However, in some cases, 30 μM $H_2O_2$ also caused spike and plateau signals. The average lag time between exposure to 10 μM $H_2O_2$ and the generation of $Ca^{2+}$ responses was 7.4 ± 1.0 min, and it tended to decrease as the concentration of $H_2O_2$ was increased (5.1 ± 0.5 min for 30 μM, 4.0 ± 0.4 min for 100 μM, and 3.2 ± 0.4 min for 300 μM). Because 30 μM $H_2O_2$ did not induce cell death (data not shown) and generally produced reliable and reversible $[Ca^{2+}]_i$ oscillations, we chose this concentration to analyze the mechanism by which peroxide-stimulated $Ca^{2+}$ signaling occurred in subsequent experiments.

*Thapsigargin-releasable, $IP_3$-sensitive $Ca^{2+}$ Stores Are Responsible for $H_2O_2$-induced $[Ca^{2+}]_i$ Oscillations*—To identify the source of the $Ca^{2+}$ mobilization, $Ca^{2+}$ was removed from the bath solution and then 30 μM $H_2O_2$ was added. As shown in Fig. 2*A*, $[Ca^{2+}]_i$ oscillations persisted in the absence of extracellular $Ca^{2+}$ ($n = 18$), suggesting that intracellular $Ca^{2+}$ stores were the main source for $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations. Depletion of the intracellular $Ca^{2+}$ stores with thapsigargin, a specific inhibitor of SERCA, prevented $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations ($n = 20$), indicating that the intracellular $Ca^{2+}$ stores responsible for the $[Ca^{2+}]_i$ oscillations were thapsigargin-sensitive (Fig. 2*B*). To examine whether the thapsigargin-sensitive intracellular $Ca^{2+}$ store was releasable by $IP_3$ receptors or ryanodine receptors, cells were exposed to 75 μM 2-APB, 20 mM caffeine ($IP_3$ receptor antagonists), or 100 μM ryanodine (a ryanodine receptor antagonist), prior to the addition of 30 μM $H_2O_2$. As shown in Fig. 2*C* and supplemental Fig. S1, 2-APB ($n = 17$) and caffeine ($n = 13$) completely prevented the generation of $[Ca^{2+}]_i$ oscillations evoked by $H_2O_2$. In contrast to this, ryanodine failed to inhibit the $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations ($n = 15$), although it effectively blocked the $[Ca^{2+}]_i$ increases induced by 500 μM caffeine, a ryanodine receptor agonist ($n = 16$; Fig. 2, *D* and *E*). These results suggest that thapsigargin-releasable, $IP_3$-sensitive $Ca^{2+}$ stores are responsible for $H_2O_2$-induced $Ca^{2+}$ mobilization.

*Activation of PLCγ1 Is Essential for the Generation of $H_2O_2$-induced $[Ca^{2+}]_i$ Oscillations*—Because $H_2O_2$ has been shown to activate PLCγ1 in some cell types (29, 30), we investigated whether PLCγ1 was phos-

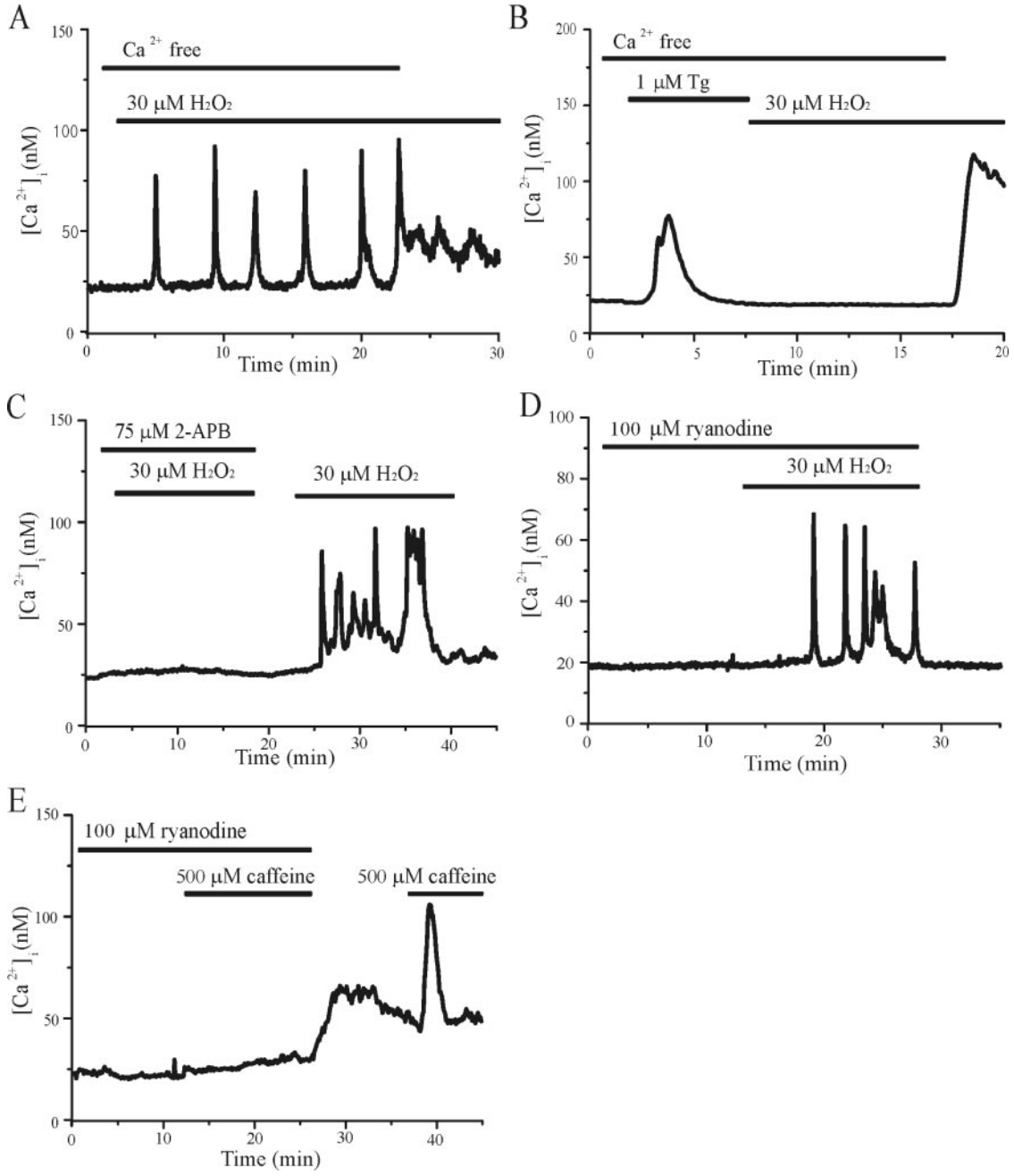


FIGURE 2. **$H_2O_2$ mobilizes $Ca^{2+}$ from thapsigargin-releasable, $IP_3$-sensitive $Ca^{2+}$ stores in cultured rat cortical astrocytes.** *A,* cells were exposed to 30 μM $H_2O_2$ in the absence of extracellular $Ca^{2+}$ as indicated by the *bars*. *B,* cells were exposed to 1 μM thapsigargin (*Tg*) followed by 30 μM $H_2O_2$ in a nominally $Ca^{2+}$-free solution. The absence of $[Ca^{2+}]_i$ increase after $H_2O_2$ application indicates that $H_2O_2$ mobilizes $Ca^{2+}$ from a thapsigargin-sensitive $Ca^{2+}$ store. *C* and *D,* $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations were prevented by 75 μM 2-APB but not by 100 μM ryanodine. *E,* ryanodine blocked the $[Ca^{2+}]_i$ increases induced by 500 μM caffeine, a ryanodine receptor agonist. For each panel, the trace is representative of 15–20 cells in three or four independent experiments.

phorylated following $H_2O_2$ stimulation of cultured rat astrocytes. PLCγ1 possesses three tyrosine residues, Tyr-771, Tyr-783, and Tyr-1254. Among them, Tyr-783 is known to be essential for $IP_3$ formation (31). Therefore, a phosphospecific tyrosine 783 antibody was used to detect the $H_2O_2$-induced phosphorylation of PLCγ1. As shown in Fig. 3, *A* and *B*, exposure of the astrocytes to various concentrations of $H_2O_2$ for 10 min induced PLCγ1 phosphorylation on tyrosine residue 783 in a dose-dependent manner ($n = 6$).

To clarify further the involvement of PLCγ1 in the generation of $[Ca^{2+}]_i$ oscillations, we used the PLC inhibitor U73122 and as control its inactive analogue U73343. As shown in Fig. 3, *C* and *D*, 10 μM U73122, but not 10 μM U73343, prevented the $H_2O_2$-evoked $[Ca^{2+}]_i$ oscillations. The frequencies of $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations were 6.5 ± 1.2 peaks/20 min ($n = 14$) in the presence of U73343 and 0.8 ± 0.8 peaks/20 min ($n = 16$) in the presence of U73122. The effect of U73122 and U73343 on the $H_2O_2$-induced PLCγ1 phosphorylation was also examined. As shown in Fig. 3, *E* and *F*, the phosphorylation of PLCγ1 induced by $H_2O_2$ was inhibited by 10 μM U73122 but not by 10 μM U73343 ($n = 3$).

The involvement of PLCγ1 was further investigated by using RNA interference (RNAi). As shown in Fig. 4, *A* and *B*, transfection of siRNA-PLCγ1 suppressed the PLCγ1 expression level, although that of PLCβ1 expression was not affected ($n = 5$). To examine the functional consequences of depletion of PLCγ1 by RNAi, cells were transfected with pSUPER (empty vector) or siRNA-PLCγ1 prior to measurement of

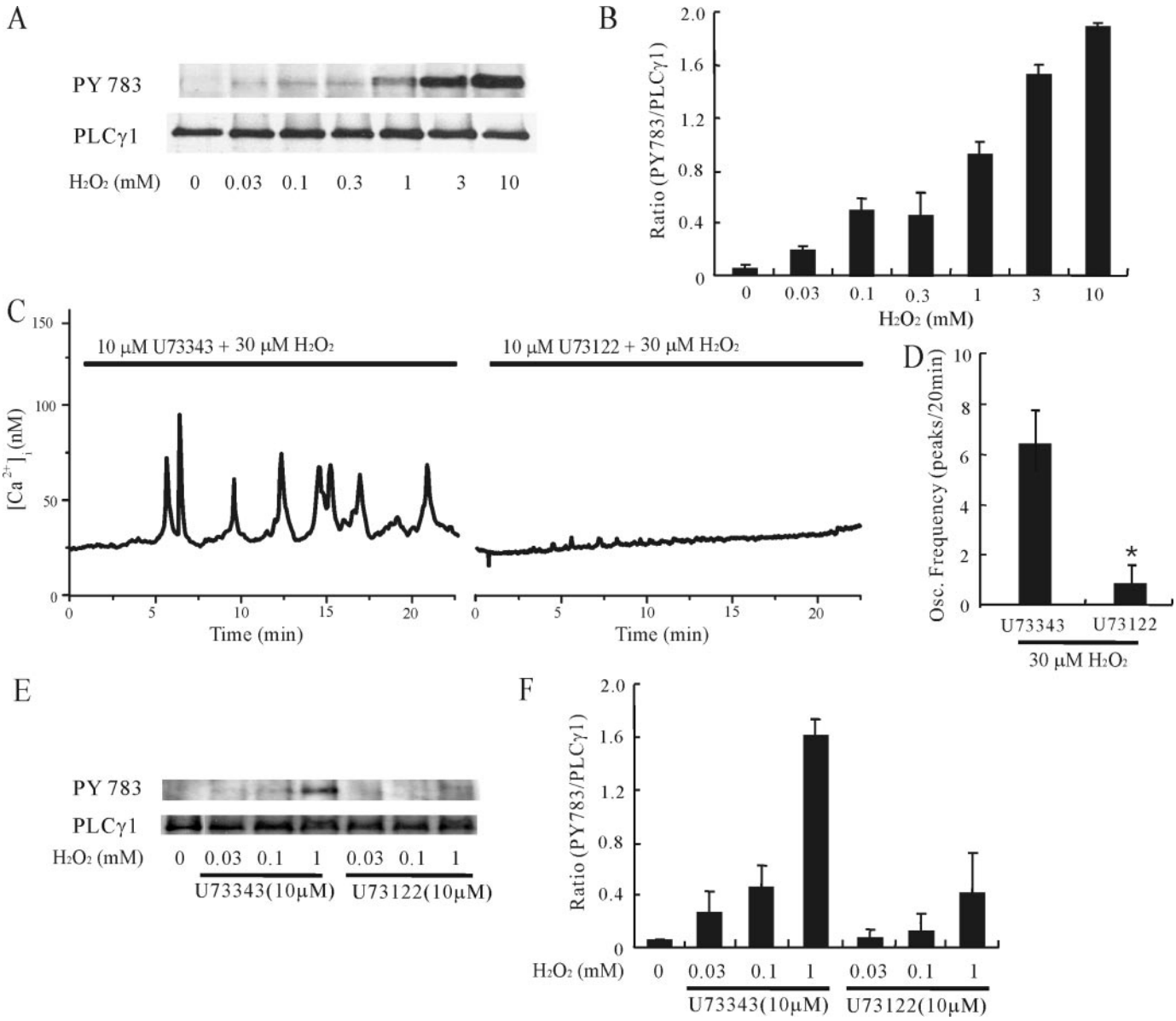


FIGURE 3. **Activation of PLCγ1 plays an essential role in $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations in cultured rat cortical astrocytes.** *A*, cells were treated with the indicated concentrations of $H_2O_2$ for 10 min, and lysates were subjected to immunoblot analysis with specific antibodies to phosphotyrosine (*PY783*) or PLCγ1. *B*, data from six experiments were quantified, and the ratio (PY783/PLCμ1) was calculated. *C*, cells were exposed to 30 μM $H_2O_2$ in combination with U73343 or U73122 as indicated by the *bars*. *D*, quantitation of results in *C*. Oscillation frequency (peaks/20 min) was counted ($n = 14–16$). * indicates the difference of the oscillation frequencies between U73343- and U73122-treated groups ($p < 0.05$). *E*, cells were treated with the indicated concentrations of $H_2O_2$ in combination with 10 μM U73343 or 10 μM U73122 for 10 min, and lysates were subjected to immunoblot analysis with specific antibodies to phosphotyrosine (*PY783*) or PLCγ1. *F*, data from three experiments were quantified, and the ratio (*PY783/PLCγ1*) was calculated. Results are presented as mean ± S.E.

$[Ca^{2+}]_i$. As shown in Fig. 4, *C* and *D*, transfection with GFP and siRNA-PLCγ1 resulted in the prevention of $Ca^{2+}$ responses to $H_2O_2$ ($n = 10$). The frequencies of $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations were 6.8 ± 0.26 peaks/20 min in GFP/PLCγ1-negative cells and 0.5 ± 0.2 peaks/20 min in GFP/PLCγ1-positive cells. Transfection with GFP and pSUPER had no effect on $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations (data not shown).

To confirm whether the transfection of siRNA-PLCγ1 had any effect on the $Ca^{2+}$ response elicited by a PLCβ-activating agonist, we stimulated the cells with histamine. As shown in Fig. 4, *E* and *F*, the cotransfection of GFP and siRNA-PLCγ1 did not prevent the $Ca^{2+}$ responses to histamine ($n = 6$). These results indicate that PLCγ1 is necessary for the generation of $[Ca^{2+}]_i$ oscillations in response to $H_2O_2$.

In addition, we also observed that expression of the $IP_3$ sponge completely abrogated $[Ca^{2+}]_i$ oscillations in response to $H_2O_2$ ($n = 8$; see Fig. 5, *A* and *B*), although the expression of low affinity $IP_3$ sponge did not prevent the $H_2O_2$-evoked $[Ca^{2+}]_i$ oscillations ($n = 6$; Fig. 5, *C* and *D*). This result strongly suggests that $IP_3$ production through the activation of PLCγ1 is a critical step for the $H_2O_2$-induced generation of $[Ca^{2+}]_i$ oscillations.

*Oxidation of a PLCγ1-associated Signaling Component Is Responsible for $H_2O_2$-induced $[Ca^{2+}]_i$ Oscillations*—To examine whether the $H_2O_2$-induced PLCγ1 phosphorylation and $[Ca^{2+}]_i$ oscillations were attributed to the sulfhydryl oxidation-dependent mechanisms, we treated cells with 1 mM DTT, a sulfhydryl-reducing agent, 4 min prior to the addition of $H_2O_2$. As shown in Fig. 6, *A* and *B*, the phosphorylation of PLCγ1 by $H_2O_2$ was prevented by pretreatment with 1 mM DTT ($n = 5$). Furthermore, the generation of $[Ca^{2+}]_i$ oscillations by 30 μM $H_2O_2$ was also inhibited by 1 mM DTT ($n = 16$; Fig. 6*C*). These data suggest that

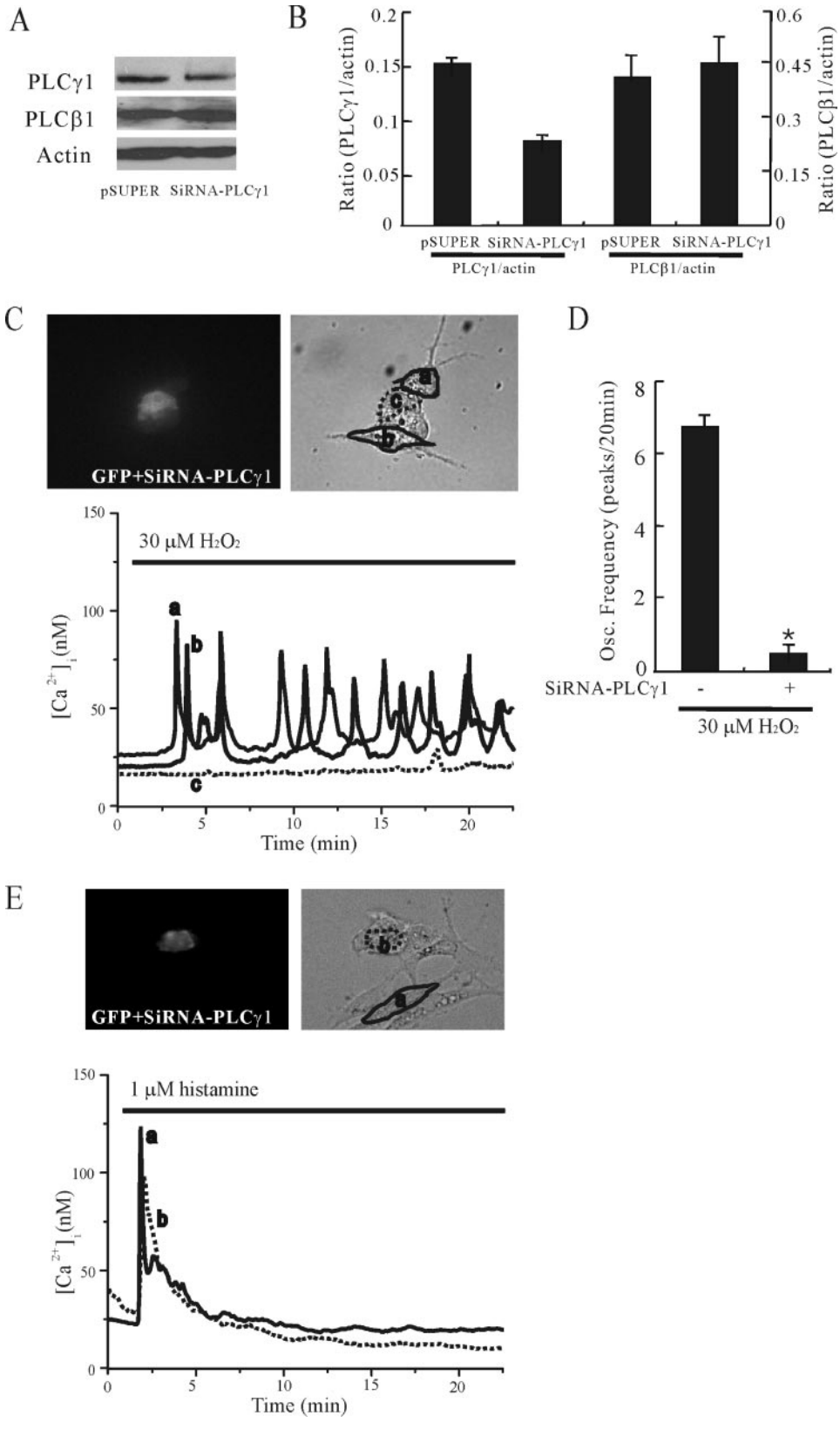


FIGURE 4. **PLCγ1 RNA interference results in the prevention of $Ca^{2+}$ responses to $H_2O_2$ in cultured rat cortical astrocytes.** *A,* Western blots of cell lysates transfected with pSUPER (empty vector) or siRNA for PLCγ1 (*siRNA-PLCγ1*). Gels were transferred and blotted with specific antibodies to PLCγ1, PLCβ1, or actin. *B,* data from four experiments were quantified, and the ratios (*PLCγ1/Actin* and *PLCβ1/Actin*) were calculated. *C* and *E,* cells were cotransfected with GFP and siRNA-PLCγ1, and $[Ca^{2+}]_i$ was measured. Note that GFP- and siRNA-PLCγ1-transfected cells (designated as *c* in *C*) exhibited a lack of $Ca^{2+}$ response to 30 μM $H_2O_2$ ($n = 8$; *C*), although GFP and siRNA-PLCγ1 transfection (designated as *b* in *E*) did not affect the $Ca^{2+}$ response to histamine ($n = 6$; *E*). *D,* the oscillation frequency (peaks/20 min) in cells transfected with or without siRNA-PLCγ1 was counted during $H_2O_2$ stimulation. Results are means ± S.E. * indicates the difference of the oscillation frequencies between siRNA-PLCγ1-transfected and nontransfected groups ($p < 0.05$).

oxidation of a PLCγ1-associated signaling component is responsible for the activation of PLCγ1 and generation of $[Ca^{2+}]_i$ oscillations by $H_2O_2$.

*$H_2O_2$ Might be Involved in the EGF-induced Activation of PLCγ1 and $Ca^{2+}$ Mobilization*—Because it has been known that EGF elevates $[Ca^{2+}]_i$ and produces $H_2O_2$ in fibroblasts (32), we investigated whether $H_2O_2$ was produced in response to EGF and if the endogenously produced $H_2O_2$ was involved in PLCγ1 activation and $[Ca^{2+}]_i$ increases in cultured rat cortical astrocytes. As shown in Fig. 7, *A* and *B*, EGF at a concentration of 10 ng/ml induced an increase in DCF fluorescence intensity that was prevented by 5 mM NAC, indicating that EGF increased an

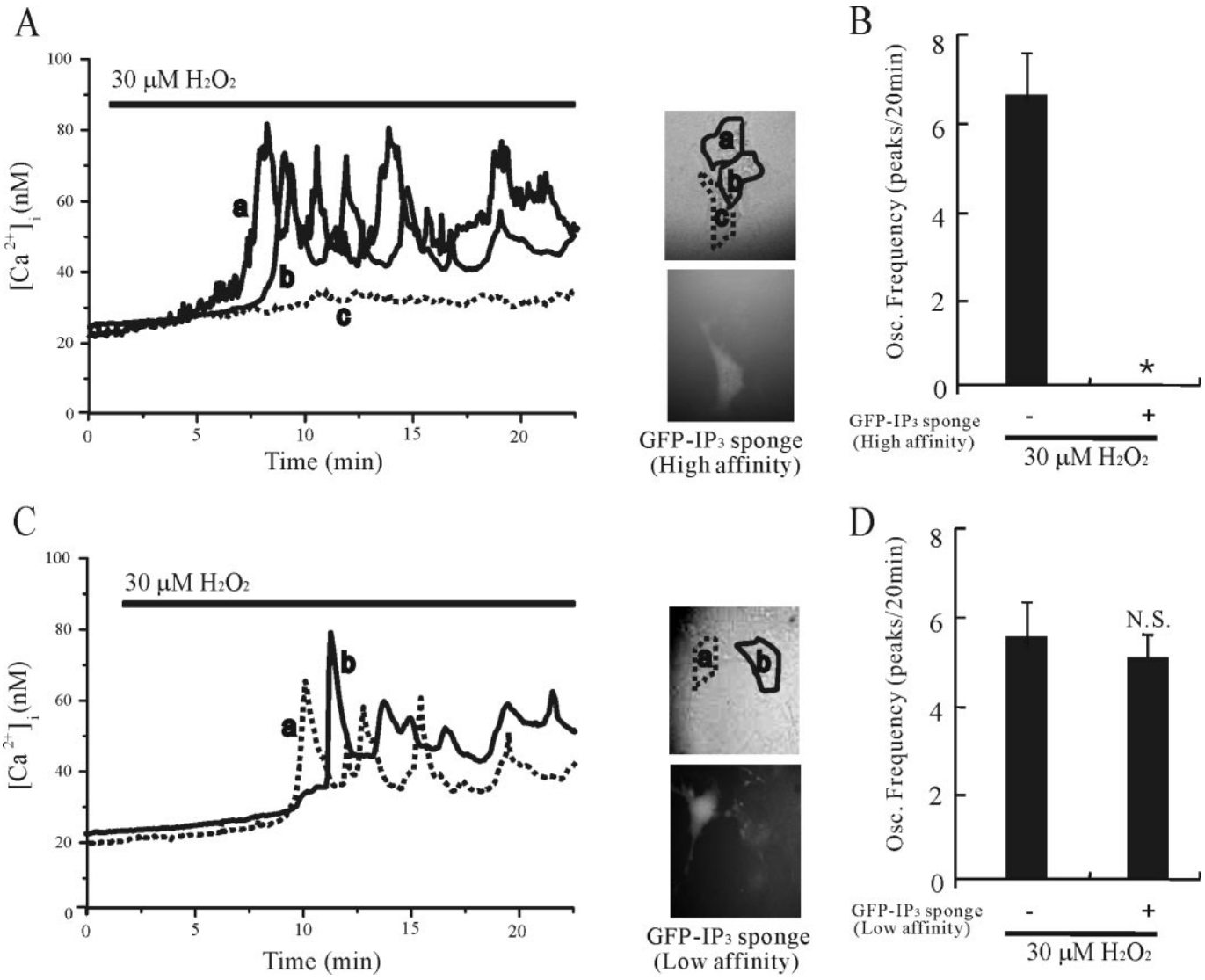


FIGURE 5. **Expression of the $IP_3$ sponge prevents $[Ca^{2+}]_i$ oscillations evoked by $H_2O_2$ in cultured rat cortical astrocytes.** Cells were transfected with GFP-tagged high affinity $IP_3$ sponge (*A*) or low affinity $IP_3$ sponge (*C*), and $[Ca^{2+}]_i$ was measured. Note that GFP-tagged high affinity $IP_3$ sponge-transfected cell (designated as *c* in *A*) exhibited the abolition of $Ca^{2+}$ response to 30 $\mu$M $H_2O_2$, whereas GFP-tagged low affinity $IP_3$ sponge-transfected cell (designated as *a* in *C*) did not. The oscillation frequencies (peaks/20 min) in cells transfected with the high affinity (*B*) and low affinity $IP_3$ sponges (*D*) were counted during $H_2O_2$ stimulation in 8 and 6 independent experiments, respectively. Results are means ± S.E. * indicates the difference of the oscillation frequencies between the transfected and nontransfected groups ($p < 0.05$). *N.S.* indicates that there is no significant difference of the oscillation frequencies between the transfected and nontransfected groups ($p > 0.05$).

accumulation of ROS ($n = 5$). The DCF fluorescence intensity was also decreased by 10 $\mu$M DPI (an inhibitor of NADPH oxidase), suggesting that NADPH oxidase, at least in part, participated in the EGF-triggered generation of ROS ($n = 5$).

EGF induced a rapid transient peak increase in $[Ca^{2+}]_i$ that subsequently declined ($n = 15$; Fig. 8*A*). However, pretreatment with 5 mM NAC or 10 $\mu$M DPI attenuated the EGF-induced $[Ca^{2+}]_i$ increases, and removal of NAC and DPI in the continued presence of EGF increased $[Ca^{2+}]_i$ again ($n = 16–20$; Fig. 8, *B* and *C*). The effect of NAC and DPI on the EGF-induced activation of PLCγ1 was also investigated. As shown in Fig. 8, *D* and *E*, the immediate strong activation of PLCγ1 followed by a sustained weak activation was observed following EGF stimulation, but in the presence of 5 mM NAC or 10 $\mu$M DPI the activation of PLCγ1 was greatly reduced ($n = 3$).

Because Prx II is a cellular peroxidase that eliminates endogenous $H_2O_2$ produced in response to growth factors such as EGF (33), we examined whether the overexpression of Prx II also attenuated EGF-induced $[Ca^{2+}]_i$ oscillations. As shown in Fig. 8, *F* and *G*, overexpression of Prx II decreased the amplitude of EGF-induced $[Ca^{2+}]_i$ increase by about 57% ($n = 6$). These data suggested that $H_2O_2$ is generated by the activation of NADPH oxidase and is subsequently involved in the activation of PLC and the elevation of $[Ca^{2+}]_i$ during EGF stimulation in cultured rat astrocytes.

## DISCUSSION

In this study, we report that exogenous addition of low concentrations of $H_2O_2$ triggers $[Ca^{2+}]_i$ oscillations through the activation of PLCγ1 in cultured rat cortical astrocytes. $H_2O_2$-mediated elevation of cytosolic $Ca^{2+}$ levels has been shown previously in various cell types (32–36). However, in many cases, $[Ca^{2+}]_i$ increases were induced by relatively high concentrations of $H_2O_2$, which are generally considered cytotoxic. The physiologically relevant concentration range of $H_2O_2$, which causes an acceleration of cellular functions in a variety of cell types, is considered 1–100 $\mu$M, although it depends on cell type (1, 37). In our system, we used 30 $\mu$M $H_2O_2$; it did not cause cell death (data not shown), and its effect on $[Ca^{2+}]_i$ was reversible, suggesting that 30 $\mu$M

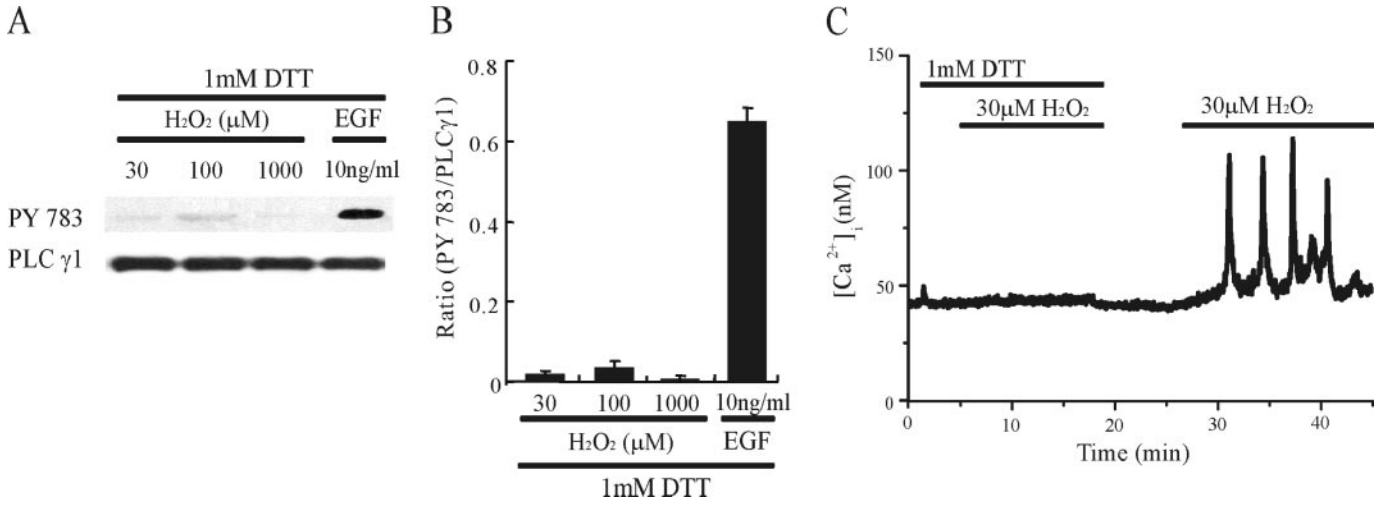


FIGURE 6. **Oxidation of a PLCγ1-associated signaling component is responsible for the $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations in cultured rat cortical astrocytes.** *A,* effect of DTT (1 mM), a sulfhydryl-reducing agent, on $H_2O_2$-induced PLCγ1 phosphorylation. Cells were incubated with DTT for 4 min followed by an addition of indicated concentrations of $H_2O_2$ (*1st* to *3rd lanes*) for 10 min or EGF (*4th lane*) for 2 min. Cell lysates were subjected to immunoblot analysis with specific antibodies to phosphotyrosine (*PY783*) or PLCγ1. *B,* quantitation of results in *A*. Ratio (*PY783/PLCγ1*) was calculated ($n = 5$). Results are presented as mean ± S.E. Note that the dose-dependent increases in the ratio (PY783/PLCγ1) by $H_2O_2$ shown in Fig. 3*B* were prevented by DTT. *C,* the $Ca^{2+}$ responses of individual astrocytes to 30 μM $H_2O_2$ in the presence or absence of 1 mM DTT are shown. Note that 1 mM DTT completely abolished $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations. The result is representative of 16 cells in three independent experiments.

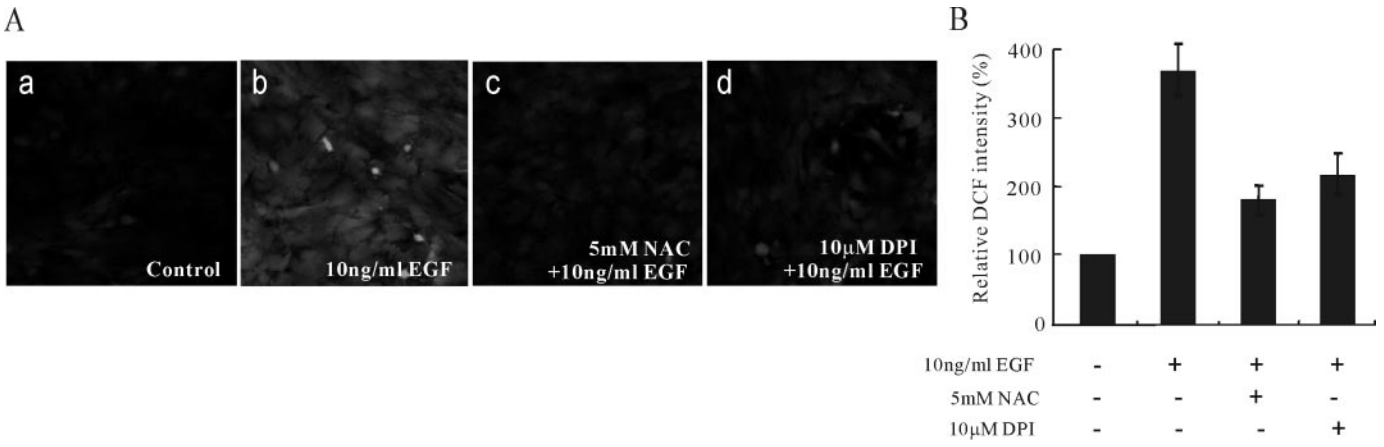


FIGURE 7. **EGF produces ROS by the activation of NADPH oxidase in cultured rat cortical astrocytes.** *A,* astrocytes were loaded with DCF for 5 min (*a–d*). Cells were treated with 5 mM NAC (*c*) or 10 μM DPI (*d*) for 2 min followed by an addition of 10 ng/ml EGF (*b–d*) for 2 min. The fluorescence of DCF was subsequently visualized by confocal laser scanning microscopy. *B,* the DCF fluorescence was quantified, and the relative intensities were calculated by setting the fluorescence intensity of control cells to 100% ($n = 5$). Results are means ± S.E.

$H_2O_2$ is close to the physiological concentration that modulates calcium signaling in astrocytes.

Cellular $Ca^{2+}$ signals generally encode information in two different modes as follows: frequency-modulated and amplitude-modulated signals (38). Frequency-modulated $Ca^{2+}$ signaling (*i.e.* $[Ca^{2+}]_i$ oscillations) is generally considered to have the highest fidelity, and many cells use this paradigm in response to low physiological concentrations of agonists (39). $H_2O_2$ has been shown to increase $[Ca^{2+}]_i$ in many cell types, but demonstrations of the generation of $[Ca^{2+}]_i$ oscillations have been rare. However, in this study we show that low concentrations of $H_2O_2$ generated $[Ca^{2+}]_i$ oscillations in astrocytes although high concentrations induced sustained increases in $[Ca^{2+}]_i$. It is more reasonable for cells to use frequency-modulated $Ca^{2+}$ signaling to avoid cell damage, especially when a prolonged period of $Ca^{2+}$ signaling is necessary. In this regard, low concentration of $H_2O_2$ is considered to be a reliable intracellular messenger involved in $Ca^{2+}$ signaling.

In most nonexcitable cells, such as astrocytes, both $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores and $Ca^{2+}$ influx through $Ca^{2+}$ channels on the plasma membrane are necessary for the generation and maintenance of $[Ca^{2+}]_i$ oscillations (40). In the present study, we demonstrate that $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations were sustained in the absence of extracellular $Ca^{2+}$, indicating that intracellular $Ca^{2+}$ stores were primarily responsible for the generation of $[Ca^{2+}]_i$ oscillations. The two main intracellular organelles containing large amounts of $Ca^{2+}$ are the endoplasmic reticulum and mitochondria (41). Previously, both of these $Ca^{2+}$ stores were shown to be involved in $H_2O_2$-induced $[Ca^{2+}]_i$ increases (17). However, our data showed that depletion of intracellular $Ca^{2+}$ stores with thapsigargin completely prevented the generation of $H_2O_2$-evoked $[Ca^{2+}]_i$ oscillations, suggesting that the thapsigargin-sensitive endoplasmic reticulum $Ca^{2+}$ store was the source of $[Ca^{2+}]_i$ oscillations.

$H_2O_2$ was also reported to be involved in the mobilization of $Ca^{2+}$ by activating intracellular $Ca^{2+}$ channels, such as ryanodine receptors and $IP_3$ receptors (15, 16). The effect of ROS on ryanodine receptors has been well established. Sulfhydryl oxidation of ryanodine receptors has been reported to activate the channels (42, 43). However, in the present study, a high concentration of ryanodine (100 μM), which blocked the $Ca^{2+}$ mobilization induced by caffeine, a ryanodine receptor agonist, failed to prevent the $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations. Instead, 2-APB and caffeine, $IP_3$-sensitive $Ca^{2+}$ channel inhibitors, blocked the $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations. 2-APB is known to have several cellular

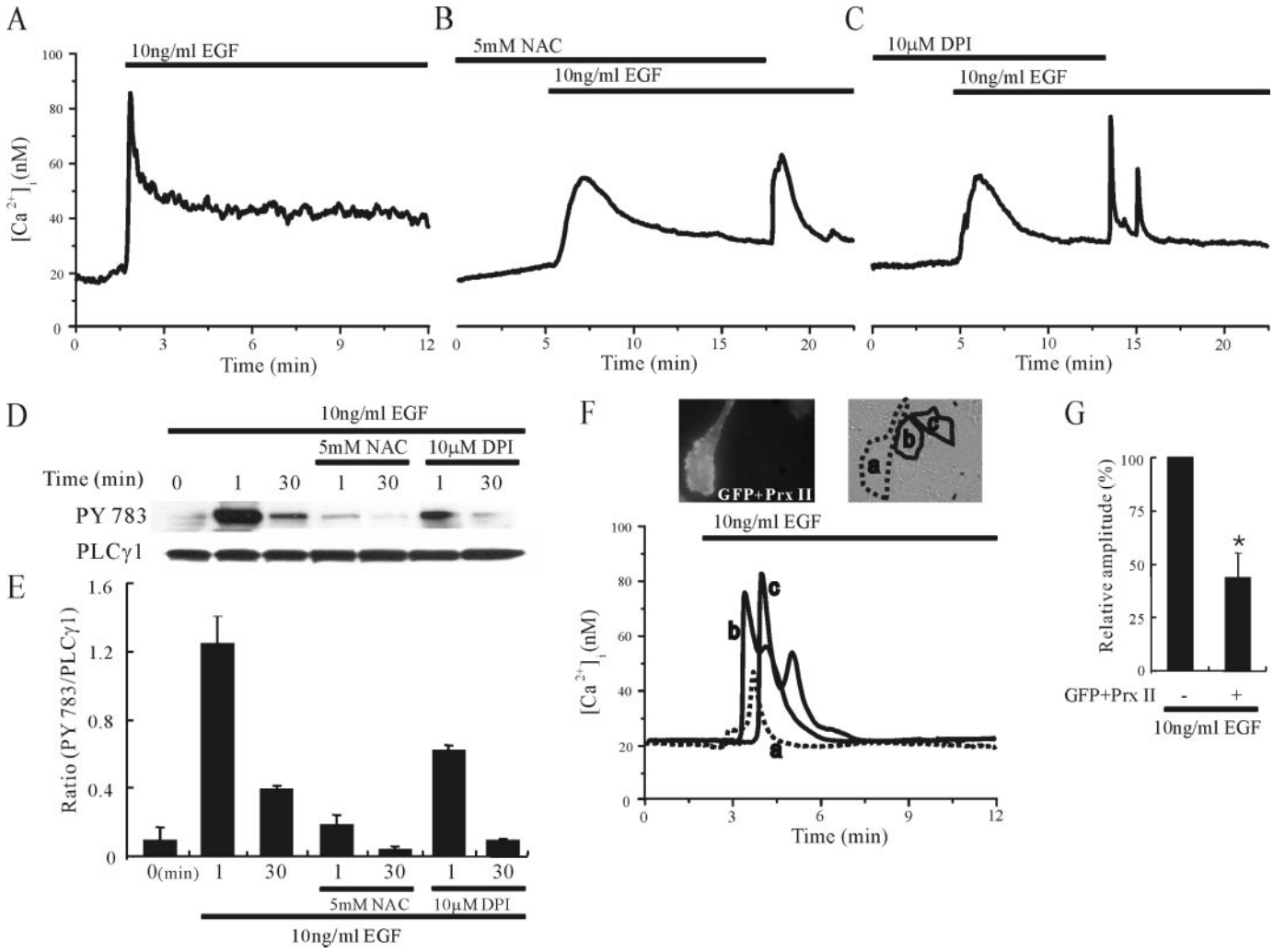


FIGURE 8. **Endogenously produced $H_2O_2$ enhances EGF-induced PLCγ1 activation and $Ca^{2+}$ mobilization in cultured rat cortical astrocytes.** The $Ca^{2+}$ responses of single astrocytes to 10 ng/ml EGF in the absence (*A*) or presence of 5 mM NAC (*B*) or 10 μM DPI (*C*) are denoted by the *bars*. Note that pretreatment with NAC or DPI suppressed $Ca^{2+}$ responses to EGF, and the removal of NAC and DPI permitted the $Ca^{2+}$ responses. Results are representative of 15–20 cells in three or four independent experiments. *D*, the effect of 5 mM NAC or 10 μM DPI on 10 ng/ml EGF-induced PLCγ1 phosphorylation is shown. Cells were incubated with or without NAC or DPI for 2 min, and then 10 ng/ml EGF was added for the indicated times. Cells were then lysed, and the lysates were subjected to immunoblot analysis with antibodies to phosphotyrosine (*PY783*) or PLCγ1. *E*, quantitation of results in *D*. Ratio (*PY783/PLCγ1*) was calculated ($n = 3$). *F*, cells were cotransfected with GFP and Prx II, which eliminates $H_2O_2$, and $[Ca^{2+}]_i$ was measured. Note that GFP- and Prx II-transfected cell (designated as *a*) exhibited reduced $Ca^{2+}$ response to EGF. *G*, the amplitude of $[Ca^{2+}]_i$ increases (nM) in cells transfected with or without GFP and Prx II was measured during EGF stimulation in six independent experiments. Results are presented as mean ± S.E. * indicates the difference of the amplitudes of $[Ca^{2+}]_i$ increases between the Prx II-transfected and nontransfected groups ($p < 0.05$).

targets; it blocks $IP_3$-sensitive $Ca^{2+}$ channels, SERCA activity, and capacitative $Ca^{2+}$ entry channels (44, 45). However, the inhibitory effect of 2-APB on $[Ca^{2+}]_i$ oscillations was unlikely to be due to the inhibition of SERCA, because the concentration of 2-APB we used in this study (75 μM) was lower than the half-maximal inhibitory concentration for SERCA (91 μM) (44). In addition, 75 μM 2-APB did not show any evidence of $[Ca^{2+}]_i$ increase when applied to itself. This is in contrast to 1 μM thapsigargin, a specific SERCA inhibitor, which induced a rapid increase in $[Ca^{2+}]_i$ as shown in Fig. 2*B*. Inhibition of SERCA has been shown to be associated with an increase in $[Ca^{2+}]_i$ in most cell types. Furthermore, it is unlikely that the effect of 2-APB on the $[Ca^{2+}]_i$ oscillations was because of inhibition of capacitative $Ca^{2+}$ entry, because as shown for the experiments performed in the absence of extracellular $Ca^{2+}$, $Ca^{2+}$ entry is not required for the oscillations.

Caffeine also has several cellular targets. It can stimulate ryanodine receptors, inhibit both cAMP degradation and PLC activation, and prevent $IP_3$-sensitive $Ca^{2+}$ channel opening. However, the only feature that caffeine and 2-APB share is their ability to antagonize $IP_3$-mediated $Ca^{2+}$ release. Therefore, although neither 2-APB nor caffeine are solely selective for $IP_3$-sensitive $Ca^{2+}$ channels, when used judiciously these pharmacological agents can be used to reveal the specific involvement of $IP_3$ signaling. The results obtained using 2-APB and caffeine support the hypothesis that $H_2O_2$ induced $[Ca^{2+}]_i$ oscillations through activation of $IP_3$-sensitive $Ca^{2+}$ channels.

$H_2O_2$ may activate signaling components responsible for $IP_3$ production. In some cell types, it has been reported that $H_2O_2$ induces an activation of PLCγ1 (29, 30). PLCγ1 is known to be recruited to the plasma membrane following activation of receptor tyrosine kinases and activated by a mechanism that relies on tyrosine phosphorylation (46). The phosphorylated PLCγ1 catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol and $IP_3$, leading to the activation of protein kinase C and increases in $[Ca^{2+}]_i$, respectively (47). Recently, PLCγ1 has been reported to be tyrosine-phosphorylated following $H_2O_2$ treatment and to protect cells from oxidant injury (18). However, the involvement of $H_2O_2$-induced PLCγ1 activation in the generation of $[Ca^{2+}]_i$ oscillations, a typical form of physiological $Ca^{2+}$

responses, has not been explored. Here, we provide evidence supporting the key role of PLCγ1 in the generation of $[Ca^{2+}]_i$ oscillations induced by low concentrations of $H_2O_2$. First, $H_2O_2$ induced dose-dependent phosphorylation of PLCγ1, which was in agreement with the previous results reported by others (18, 29). Second, 30 μM $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations were almost completely prevented by U73122 but not by U73343. Third, RNAi against PLCγ1 inhibited $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations. Finally, expression of the $IP_3$ sponge completely abolished $[Ca^{2+}]_i$ oscillations in response to $H_2O_2$. These results suggest that the production of $IP_3$ following the activation of PLCγ1 is essential for generation of $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations in cultured rat cortical astrocytes. In addition to this, we showed that the $H_2O_2$-induced phosphorylation of PLCγ1 and $[Ca^{2+}]_i$ oscillations were prevented by DTT, a sulfhydryl-reducing agent. Therefore, the oxidation of a PLCγ1-associated signaling component appeared to be responsible for the $H_2O_2$-induced $[Ca^{2+}]_i$ oscillations.

Although the activation of PLCγ1 was shown to play a critical role in the 30 μM $H_2O_2$-evoked generation of $[Ca^{2+}]_i$ oscillations in our system, we do not rule out the possibility that $H_2O_2$ also increases the sensitivity of $IP_3$ receptors to $IP_3$ in this cell type. Previously, thimerosal, which catalyzes the oxidation of thiol groups, was reported to trigger $[Ca^{2+}]_i$ oscillations by increasing the affinity of $IP_3$ receptors for $IP_3$ (46, 49, 50). In addition, Hu *et al.* (16, 51) showed that NADPH oxidase-derived $H_2O_2$ increased the sensitivity of intracellular $Ca^{2+}$ stores to $IP_3$ and played a critical role in generating $[Ca^{2+}]_i$ oscillations in human endothelial cells stimulated by histamine. Taken together, our study and those of Hu *et al.* (16, 51) imply that both PLCγ1 activation and increased sensitivity of $IP_3$ receptors may contribute to the generation of $[Ca^{2+}]_i$ oscillations.

By having observed the stimulatory effect of exogenous $H_2O_2$ on intracellular $Ca^{2+}$ signaling, we sought to determine whether $H_2O_2$ was produced by receptor stimulation and if endogenously generated $H_2O_2$ played a modulatory role in $Ca^{2+}$ signaling in rat astrocytes. Stimulation of EGF receptors has been shown previously to induce both $H_2O_2$ production and $[Ca^{2+}]_i$ increases in fibroblasts (32). The EGF receptor belongs to a family of transmembrane receptors with intrinsic tyrosine kinase activity (52). The production of intracellular $H_2O_2$ in response to EGF was shown to require the activation of the Rac-NADPH oxidase signaling, whereas activation of PLCγ1 was regarded to be critical for $[Ca^{2+}]_i$ increases (53–55). Although Rac was suggested to play a role in the EGF-induced $Ca^{2+}$ signaling (32, 56), the role of $H_2O_2$ in PLCγ1 activation during EGF stimulation has not been elucidated.

In this study, we showed that EGF receptor stimulation induced ROS production, PLCγ1 activation, and $[Ca^{2+}]_i$ elevation, which were all attenuated by the pretreatment with NAC, an ROS scavenger, or DPI, an NADPH oxidase inhibitor. These results indicated that ROS produced via NADPH oxidase during EGF stimulation played a critical role in the enhancement and maintenance of PLCγ1 and $Ca^{2+}$ responses in rat cortical astrocytes. As far as we know, this is the first report to show the involvement of NADPH oxidase in EGF-mediated ROS generation and the regulatory role of endogenously produced ROS in PLCγ1-activated $Ca^{2+}$ signaling in astrocytes. Previous studies have revealed that the predominant member of ROS produced by EGF stimulation was $H_2O_2$, which played a key role in the EGF-induced protein tyrosine phosphorylation and $[Ca^{2+}]_i$ increases (4, 32). Furthermore, we found that overexpression of Prx II, which probably plays an important role in eliminating $H_2O_2$ generated in the cytosol, reduced the amplitude of $[Ca^{2+}]_i$ increase evoked by EGF. Therefore, it is likely that the major component of ROS, which is produced by EGF stimulation and responsible for PLCγ1 activation and $[Ca^{2+}]_i$ elevation in our system, is $H_2O_2$. Considering that the activation of EGF receptors stimulates the proliferation and differentiation of astrocytes (57, 58), the role of $H_2O_2$ in the regulation of $Ca^{2+}$ signaling may be of physiological importance in this cell type.

We therefore conclude that physiologically relevant, low concentrations of $H_2O_2$ trigger the generation of $[Ca^{2+}]_i$ oscillations by activating PLCγ1 through sulfhydryl oxidation-dependent mechanisms in cultured rat cortical astrocytes. Given that $H_2O_2$ is a small and diffusible molecule that is produced endogenously via NADPH oxidase during EGF receptor stimulation and is involved in the enhancement of $Ca^{2+}$ signaling, $H_2O_2$ may be of physiological importance in regulating various cellular functions such as cell proliferation.

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